

In re application of
Nygren et al.
Application No. 09/982,658
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Atty. Dkt. No. 074022-2302

these modified paragraphs reflect the amendments in red ink. The sequence listing and CRF therefor are enclosed. The amendments to the application add no new matter.

In connection with the Sequence Listing submitted concurrently herewith, the undersigned hereby states that:

1. the content of the attached paper copy and the enclosed computer readable copy of the Sequence Listing, submitted in accordance with 37 C.F.R. § 1.821(c) and (e), respectively, are the same; and
2. the submission, filed herewith in accordance with 37 C.F.R. § 1.821(g), does not include new matter.

Respectfully submitted,

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methanol, and 50% isopropanol was prepared. A 400 μ l sample of this solution was applied to the wafer which was attached to a spin coating apparatus and spinning at 3000 rpm. The wafer is spun until a uniform thin film is generated. The glass is then annealed or cured onto the wafer by incubating for 2 hours at 155°C.

Surface 3: Si_3N_4

A 499 +/- 2 Å layer of silicon nitride was applied to the virgin silicon wafer using standard vapor deposition processes known in the semi - conductor industry.

Surface 4: RT II

A 1:1 mixture of the T-Polymer solution with the R-polymer solution was prepared. A 400 μ l sample of this mixture was applied to the wafer which was spinning at 3000 rpm. The wafer was spun until a uniform thin film was generated. The polymer layer is cured onto the wafer by incubating for 2 hours at 155°C.

DNA capture probe was coated onto these wafer surfaces from a solution containing 50 mM sodium citrate, pH 6.0, 0.1 mg/ml carrier DNA, sheared herring sperm DNA, and 600 μ M biotinylated DNA, 26-mer. The probe sequence was 5'-CGCTAATATCAGAGAGATAACCCAC-3'. Wafers were incubated in this solution overnight at 4°C. Wafers were removed from the solution and washed with 1x phosphate buffered saline containing 0.2% Tween 20™ detergent (PBS/Tween). The wafers were then coated in a BSA (bovine serum albumin) solution for 3 hours at 65°C. The wafers were then rinsed with PBS/Tween detergent.

To measure the amount of biotinylated DNA adsorbed to the surface, the wafers were incubated for 30 minutes with a solution containing streptavidin conjugated to horseradish peroxidase (Immunology Products) was diluted 1:250 in 50 mM MOPS, pH 7.0, containing 3% alkaline tre EEN20 detergent, and 0.5%

Proclin 300 (an anti-bacterial agent). Wafers were then washed with deionized water and dried under a stream of nitrogen. A drop of TMB precipitating substrate was applied to the surface and the wafers incubated for 30 minutes at room temperature. Thickness increases (in Angstroms) due to substrate deposition were measured using an absolute ellipsometer (Gaertner) which was normalized to the initial substrate thickness. See Figure 1. These experiments were repeated using alkaline phosphatase conjugated to streptavidin and BCIP/nitroblue tetrazolium substrate pair. The R-polymer surface (most polar surface used) and the T-polymer surface (least polar surface used) all performed well. These experiments demonstrate that DNA can be successfully immobilized to the surface of the silicon wafer.

EXAMPLE 2: Sensitivity Evaluation In An Un-optimized Assay

Wafers coated with T-polymer (see Example 1) were coated for 56 hours at 4°C in a solution containing 50 mM sodium citrate buffer, pH 6.0, 5X SSC, and 20 µg/ml of the ssDNA capture probe complimentary to M13mp18. The probe sequence was CGCTAATATCAGAGAGATAACCCAC (SEQ ID NO. 1). The coated wafers were removed from coating solution and placed into a blocking solution containing 5X Denhardt's solution, 0.5% SDS, 1 mg/ml carrier DNA, and 25 mM buffer at pH 6.5. They were incubated 16-18 hours at 4°C and then rinsed with phosphate buffered saline containing 0.0005% TWEEN20 detergent at pH 7.4. Capture probe coated wafers were hybridized with M13mp18 plasmid overnight at 60°C in a solution containing 1X Denhardt's solution, 0.5% SDS, 25 mM MES, pH 6.5, 0.2 mg/ml carrier DNA, 5X SSC, a final concentration of M13mp18 was 500 ng/ml, 1 ng/ml or 100 pg/ml. The final hybridization step occurred under the same solution and incubation conditions as the previous step with a final biotinylated amplifying probe concentration of 92 µM. The amplifying probe contains

strand sequence from 6249 to 6273 and was biotinylated at residue 6261. The sequence is GCAGGTCGACTGTAGCAGGATGCCGG. All appropriate controls were performed. Wafers were incubated with a streptavidin alkaline phosphatase conjugate. Precipitating substrate, BCIP/nitroblue tetrazolium, was used to generate an increase in thickness at the surface of the wafer. Thickness increases were measured using an absolute ellipsometer (Gaertner). Results for the experiment are shown in Figure 2. From this experiment, it was concluded that a sensitivity of 1 ng/ml and potentially as low as 100 pg/ml was achieved. This translates to a copy number of roughly 10^{10} for a very un-optimized assay.

(SEQ
ID NO.
2)

15 **EXAMPLE 3: Spin Coating of the DNA Capture Probe**

Spin coating of the DNA capture probe (M13mp18 fragment 6249-6273) was carried out on two wafer polymer preparations: T-polymer and R-polymer. The wafers were prepared as previously described in Example 1. The DNA capture probe was applied using 400 μ l of solution and a speed of 4000 rpm. Three coating solutions were prepared as follows:

High Salt Solution:

25 5 μ l of 10 mg/ml sheared herring sperm DNA
 25 μ l of 0.50 M Pipes pH 7.0 buffer
 105 μ l of methanol (anhydrous)
 315 μ l of biotinylated DNA stock (316 μ g/ml)
 50 μ l 20X SSC

Medium Salt Solution:

30 5 μ l of 10 mg/ml sheared herring sperm DNA
 25 μ l of 0.50 M Pipes pH 7.0 buffer
 105 μ l of methanol (anhydrous)
 315 μ l of biotinylated DNA stock (316 μ g/ml)
 25 μ l 20X SSC
35 25 μ l H₂O

coating solution was the most critical variable. The optimal coating solution was determined to be 0.25 mg/ml of carrier DNA, 6X SSC pH 7.5, room temperature incubation, and the best surface was the T-Polymer.

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EXAMPLE 4: Adherence of Covalently Bound DNA Under Stringent Conditions

Virgin silicon wafers were coated with a layer of film forming latex consisting of free carboxylic acid groups. A 30% stock solution of TC7A (Seradyn, Indianapolis, IN) was diluted to a 0.5% solid in methanol. A 300 microliter sample was applied to the substrate using the spin coating technique and was cured at 37°C for 120 minutes prior to use. A final thickness of this material is preferred to be 240Å. These carboxylic acid groups were reacted with NHS (20 mM), N,N'-dicyclohexylcarbodiimide (DCC, 20 mM) in dioxane. These components were mixed on ice for 15 minutes and then incubated for 4-5 hours at room temperature. Then 32 mmols of hydrazine was added to leave a reactive hydrazide on the surface. The mixture was incubated for 1 hour on ice and then overnight at room temperature. A DNA capture probe with a 3' terminal ribonucleotide residue was treated with sodium periodate at a DNA:NaIO₄ ratio of 1:15 for 1 hour to leave a dialdehyde group on the polynucleotide. The dialdehyde then adds to the carbonyl surface to form a covalent adduct of the capture probe on to the surface.

An 18 mer DNA/RNA chimera was utilized as the capture probe. The probe was biotinylated at the 5' end and has a ribonucleotide cytosine (on the 3' end) The DNA sequence was 5'-CGAAGCTTGGATCCGCC-3' (ribose). The covalently attached capture probe was treated with S1 nuclease to degrade the entire probe from the surface. The S1 nuclease was mixed in a solution of 0.2 mM NaCl, 0.05 M sodium acetate pH 5.4, 1 mM ZnSO₄, and 0.5% glycerol to a final concentration of 2 units/ml. A section of the

wafer was submerged into 7 ml of the enzyme solution and incubated for 10 minutes at 37°C. Wafers were rinsed prior to enzyme treatment in water for 2 hours at 45°C. The enzyme solution was decanted into test tubes and a small volume of water used to rinse the wafers. The combined solution was dried with a SpeedVac system. The pellet was extracted into acetone and the solution dried. These pellets were re-suspended in 70 μ l of water and the A_{260} measured in a microcuvette. The surface density of the probe was determined to be 50 ng/cm². Control surfaces where no NaIO₄ was used, no covalent attachment, did not generate signal. Control surfaces without DNA or without S1 nuclease gave no signal.

Wafers with covalently attached capture DNA probe were incubated at 68°C in pure water for 4, 7, or 24 hours in order to simulate very extreme conditions of stringency. Wafers were removed, dried and incubated with a streptavidin HRP conjugate. Unbound conjugate was rinsed from the surface. The Kirkegaard and Perry two component TMB substrate was applied for 20 minutes at room temperature. After four hours, the intensity of the ELISA was not changed, indicating that the capture DNA probe on the surface had not been stripped to any significant extent. A wafer with adsorbed, non-covalently bound DNA probe was shown to lose all of its DNA probe under these conditions. See Figure 4.

Example 5: Estimated Sensitivity Based On Bound Capture Probe

30 An 8-mer was synthesized and was 5' biotinylated and had a 3' ribose. The DNA sequence was 5'-AAAGATGTA (SEQ ID NO. 4) ribose-3'. The 8-mer was immobilized using the 15:1 periodate:probe ratio to a TC7 coated optical substrate as described in Example 4. Chips were coated at the concentrations listed in the table below. The amount of immobilized biotinylated DNA was measured by reacting a pre-determined surface area with a sufficient volume of